BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

We, Dr. Tatsuiji Seki and Dr. Kazuhito Fujiyama, declare and state:

1. We are joint inventors of the subject matter claimed in the above-identified patent application. In our patent application we demonstrated the presence of galactose on *N*-linked glycan structures in transformed tobacco cells expressing human beta-1,4-galactosyltransferase (hGT).

2. We have introduced mammalian transferases into plant cells, which were regenerated into whole plants. We transformed *Nicotiana tabacum* L. cv SR1 using *Agrobacterium tumefaciens* strain EHA101 carrying pGAhGT [a plasmid comprising the coding sequence for human beta-1,4-galactosyltransferase (hGT)]. Transformants were screened on Linsmeier and Skoog medium including kanamycin. Ninety (90) transformants were kanamycin-resistant. All transformants were tested for possibility of beta-1,4-linked galactose addition to endogenous glycoproteins using horseradish peroxidase-conjugated RCA120 lectin. Seventeen (17) transgenic tobacco plants gave RCA120 positive results suggesting the presence of beta-1,4-linked galactose.

3. To confirm the presence of beta-1,4-linked galactose on glycoproteins, we further examined glycan structures of glycoproteins from plant H133B (regenerated transgenic tobacco plant). Sugar chains prepared from glycoproteins were labeled with 2-aminopyridine (PA). The PA-oligosaccharide was incubated in 0.5M acetate buffer in the presence or absence of beta-galactosidase at 37ºC for 12 hours followed by boiling. Then the solution was fractionated on reverse phase high performance liquid chromatography (RP-HPLC) (see at Tab A, Part A). Digestion of PA-sugar chains with beta-galactosidase gave a distinct HPLC pattern, where new peaks appeared as are marked by arrows (see at Tab A, Part B). The elution positions of Peaks A and B (see at Tab A, Part C) were identical with standards for GnM3 and GnM5, respectively. Digestion of GalGnM3 and GalGnM5 with beta-galactosidase yielded GnM3 and GnM5, respectively. These results demonstrate that plant H133B held galactosylated glycan structures, for instance GalGnM3 and GalGnM5. Similarly, tobacco BY2 cell line

when transformed by human beta-1,4-galactosyltransferase gene made galactosylated glycan structures, namely, GalGnM3 and GalGnM5 (Palacpac, *et al.*, PNAS 96:4692, 1999), which results, although later than our filing date, confirm and are consistent with these results.

4. We also examined glycan structures of glycoproteins from plant H118D. PA-oligosaccharide was fractionated on RP-HPLC. All peaks were tested by beta-galactosidase digestion. The HPLC patterns of non-digested and digested samples were compared. Peaks A, B and C (see, upper panel [non-digested sample] and lower panel [digested sample] of the Figure at Tab B) were shifted to lower molecular masses. Analysis by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry using a Voyager DE™-Pro mass spectrometer showed that the mass to charge ratio (m/z) of PA-sugar chains for Peaks A, B and C were 1351.79, 1514.71 and 1677.36, respectively. These masses were assigned to the structures for GalGnM3 (1354.27), GalGnM4 (1516.41) and GalGnM5 (1678.55), respectively.

5. PA-oligosaccharides fractionated on RP-HPLC were purified by size fractionation high performance liquid chromatography (SF-HPLC). Every SF-HPLC fraction was analyzed by MALDI-TOF mass spectrometry using a Voyager DE™-Pro mass spectrometer. Sugar chains with mass possibly corresponding to galactosylated structures were digested with beta-galactosidase, suggesting that Peaks 3-4, 6-1, 8-4 and 9-3 (see, Tab C) have galactosylated structures. Sugar chains in Peak 8-4 (see, Tab C) were digested with beta-galactosidase and were purified by SF-HPLC. The purified PA-oligosaccharides were analyzed by MALDI-TOF mass spectrometry using a Voyager DE™-Pro mass spectrometer and the beta-galactosidase products had m/z of 1539.31 and 1555.16, which corresponded to Na^+ and K^+ of GnM5 (1516.41). Analysis by MALDI-TOF mass spectrometry showed that Peak 8-4 (see, Tab D) is GalGnM5 because it showed a m/z of 1679.97. Elution of Peak 8-4 on RP-HPLC was identical with synthetic

GalGnM5 (Palacpac, *et al.*, PNAS, 96:4692, 1999). All these data indicate that Peak 8-4 is GalGnM5, thereby supporting the conclusion that galactose is being appropriately added to the *N*-linked glycan structures.

6. The structures analyzed from hGT transgenic tobacco plants are shown at Tab E. Similar data generated from transgenic tomato plants is shown at Tab F. These tomato plants were produced using methods analogous to those described in Paragraph 2 above. These tables show the deduced sugar chain structures from the analyzed fractions obtained by the method of Paragraph 5.

7. The glycosylation pattern observed in the transfected plants was not the result of endogenous plant glycosylation pathways. We analyzed untransfected plants (as a control) by HPLC for the presence of *N*-linked glycan structures. The sugar chains of glycoproteins from the untransfected tobacco control plants were prepared by hydrazinolysis and labeled with 2-aminopyridine (PA). The resulting PA-labeled sugar chains were purified and characterized by a combination of RP- and SF-HPLCs. Each collected fraction (1 ~ 10) was rechromatographed by SF-HPLC. A total of 73 peaks, possibly containing the *N*-linked glycans, were observed from fractions by SF-HPLC, and then the samples in every fraction were subjected to analysis by MALDI-TOF mass-spectrometry. Analysis by MALDI-TOF mass-spectrometry showed that 30 fractions did not contain *N*-linked glycans. The RP-HPLC analysis of the remaining 43 peaks that were analyzed after being digested with several exo-glycosidases demonstrated that compounds contained in these peaks were also not *N*-linked glycans. A summary of the data is shown at Tab G, providing the deduced sugar chain structures of each fraction along with the derived and calculated mass spectrum readings.

8. Therefore, the data for whole plants shows similar results for *N*-linked glycan structures to that obtained for plant cells present in the above-identified patent application.

9. Additionally, these results demonstrate that glycosyltransferase, shown by galactosyltransferase and hGT, when expressed in mature plants, modifies the structure of *N*-linked glycans in a manner similar to that seen in mammalian cell systems.

I, Tatsuji Seki, certify, attest and swear that the foregoing statements are correct and complete.

Dated: 29 Sep 2003

Tatsuji Seki

Subscribed and sworn before me on this _____ day of _____, 2003.

_____ _____

Notary Seal

I, Kazuhito Fujiyama, certify, attest and swear that the foregoing statements are correct and complete.

Dated: Oct 20, 2003

Kazuhito Fujiyama

Subscribed and sworn before me on this 20th day of October, 2003.

Notary

OFFICIAL SEAL
SUSAN MUNOZ
NOTARY PUBLIC-ARIZONA
MARICOPA COUNTY
My Comm. Expires Dec. 4, 2006

Seal

登簿平成１５年第　963　号

嘱託人　関　達治 ――――――――――――――――――は、

当公証人の面前でこの証書に自ら　署　名　した。―

よってこれを認証する。――――――――――――――

平成１５年　９　月　２９　日当公証人役場において

大阪市中央区平野町２丁目１番２号（沢の鶴ビル内）

大阪法務局所属

公証人　堀川和男

OSAKA LEGAL AFFAIRS BUREAU
"NOTARY"
1-2, HIRANO-MACHI, 2-CHOME,
CHUO-KU, OSAKA JAPAN

大阪法務局所属　公証人役場

Registered No. 963 -2003

NOTARIAL CERTIFICATE

This is to certify that Mr.Tatsuji SEKI has affixed his signature in my very presence to the attached document.

Dated this 29th day of September, 2003

Kazuo Horikawa

KAZUO HORIKAWA

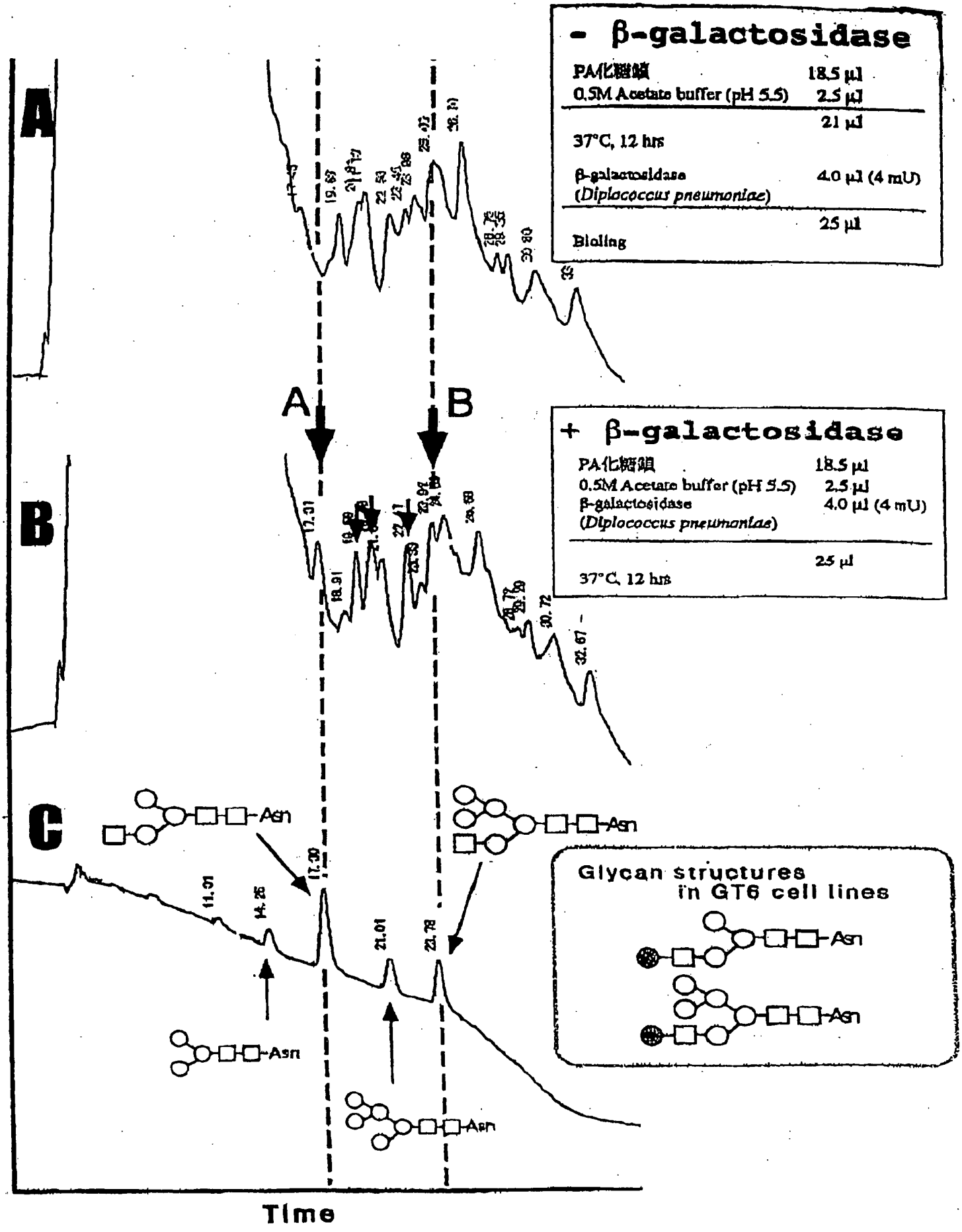
NOTARY

Osaka Legal Affairs Bureau

1 - 2, Hirano-Machi, 2- Chome

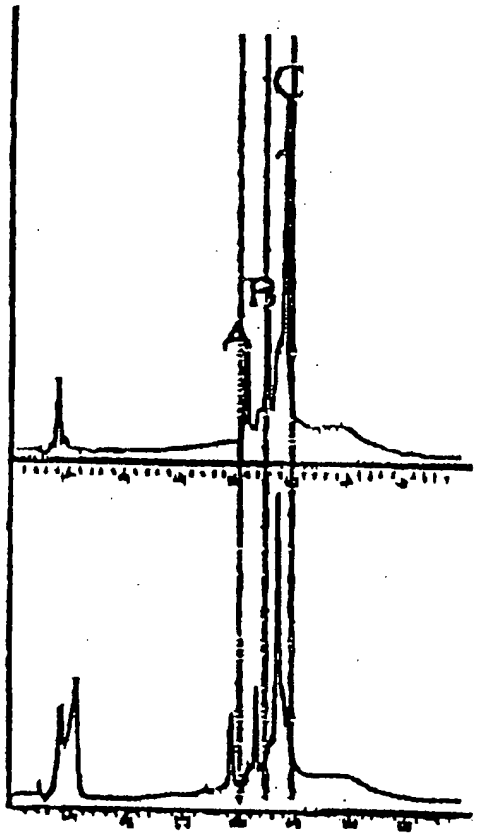
Chuo-ku, Osaka Japan

OSAKA LEGAL AFFAIRS BUREAU
"NOTARY"
1-2, HIRANO-MACHI, 2-CHOME,
CHUO-KU, OSAKA JAPAN

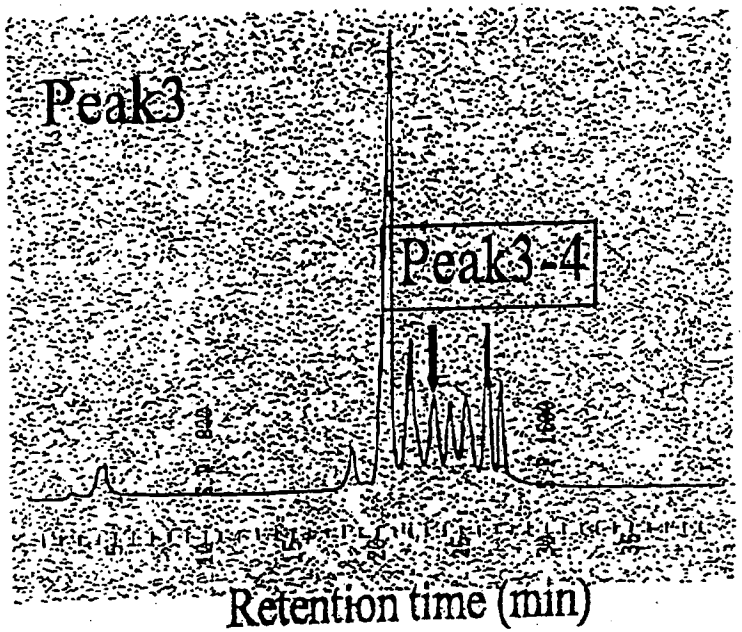

- β-galactosidase
PA化糖鎖 18.5 μl
0.5M Acetate buffer (pH 5.5) 2.5 μl
21 μl
37°C, 12 hrs
β-galactosidase 4.0 μl (4 mU)
(Diplococcus pneumoniae)
25 μl
Boiling
+ β-galactosidase
PA化糖鎖 18.5 μl
0.5M Acetate buffer (pH 5.5) 2.5 μl
β-galactosidase 4.0 μl (4 mU)
(Diplococcus pneumoniae)
25 μl
37°C, 12 hrs
A
B
C
Asn
Glycan structures
in GT6 cell lines
Time

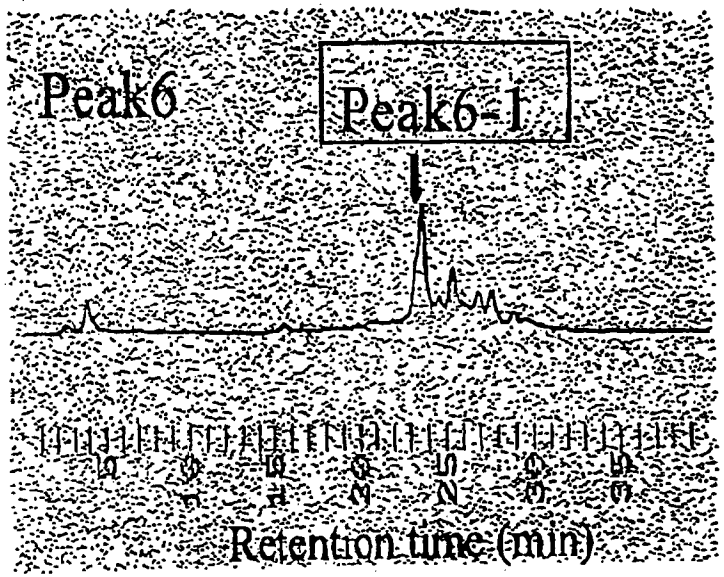
UPPER

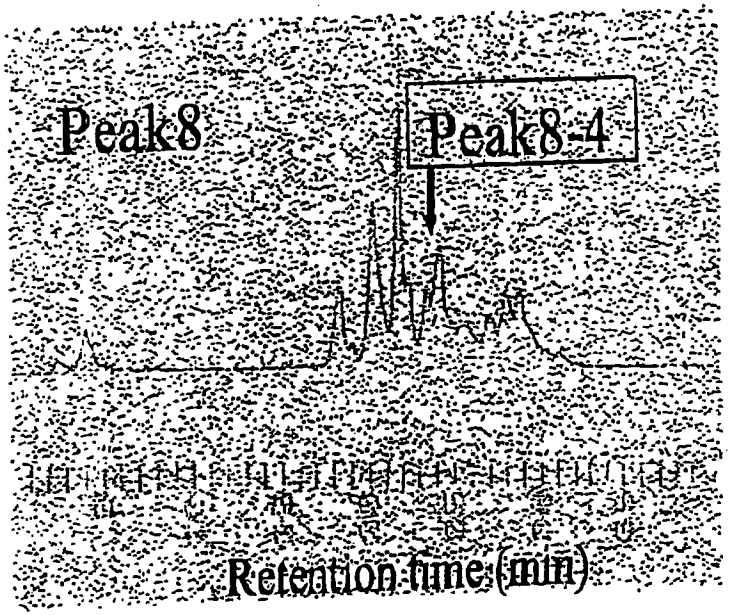
LOWER

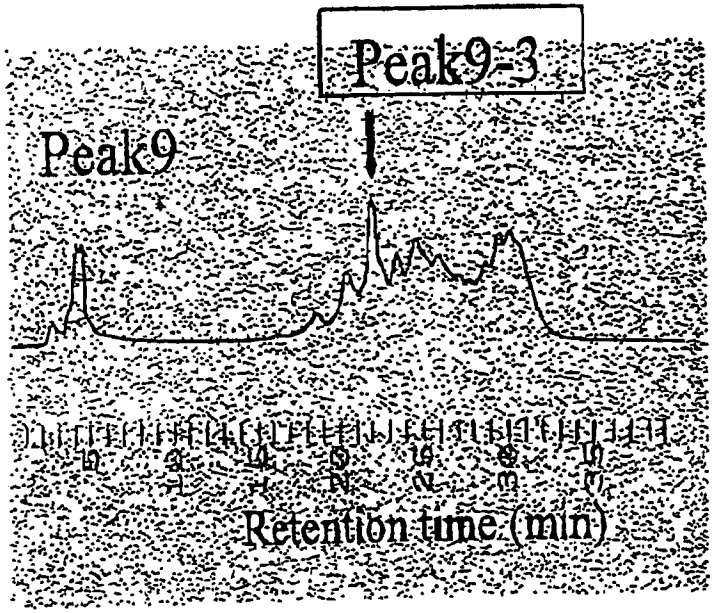


SF-HPLC


Peak3
Peak3-4
Retention time (min)

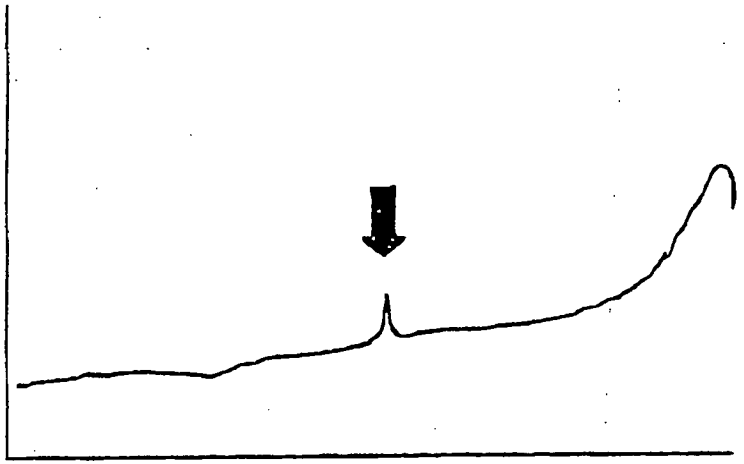

Peak6
Peak6-1
Retention time (min)


Peak8
Peak8-4
Retention time (min)


Peak9
Peak9-3
Retention time (min)

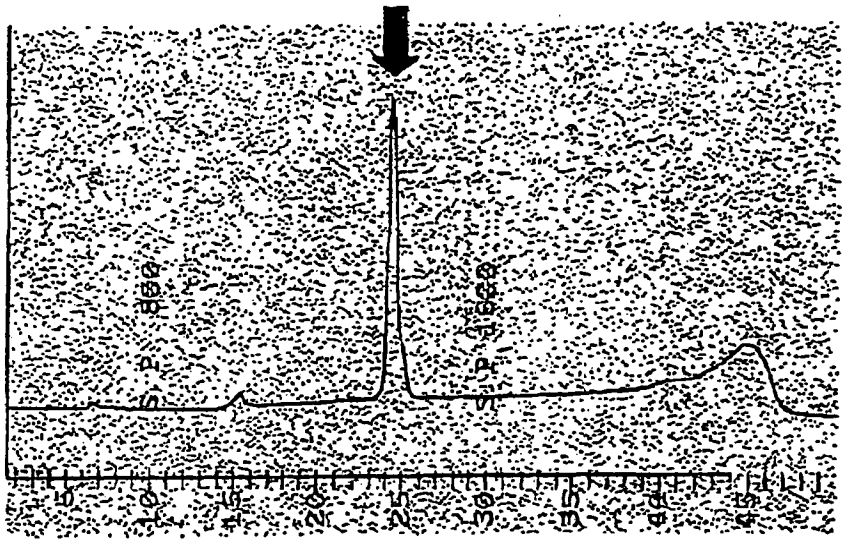
RP-HPLC

STANDARD; GalGNM5

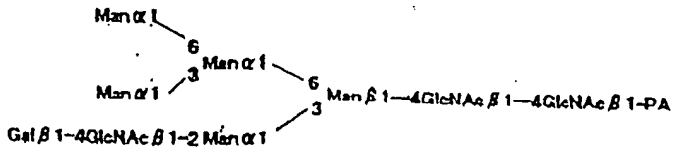
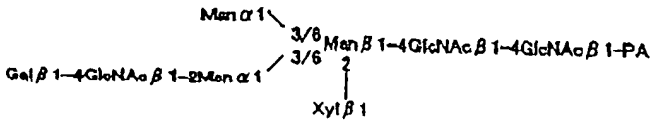
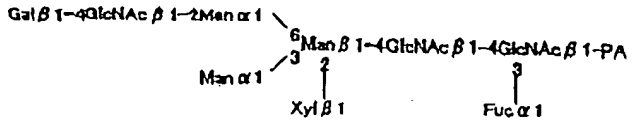
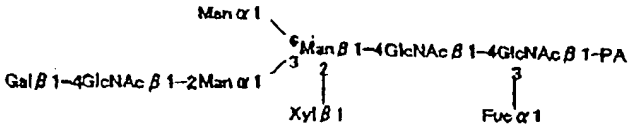
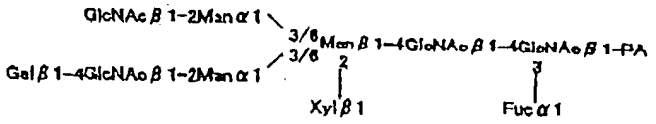
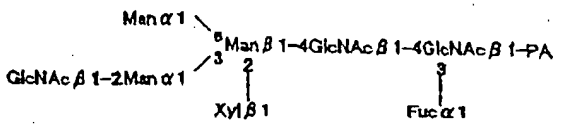
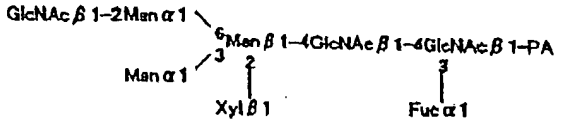
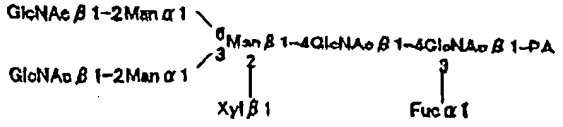
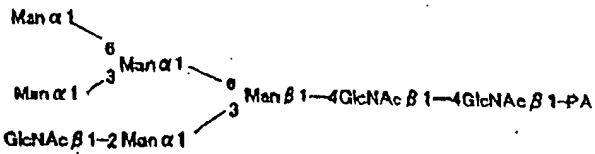


Retention time (min)

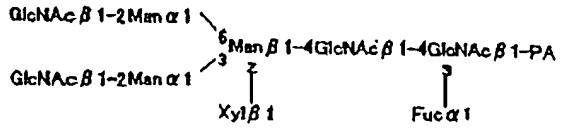
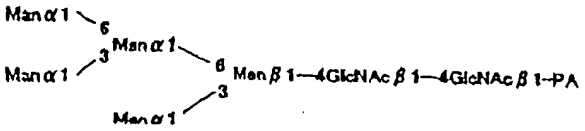
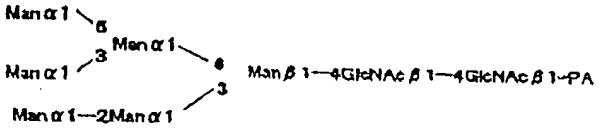
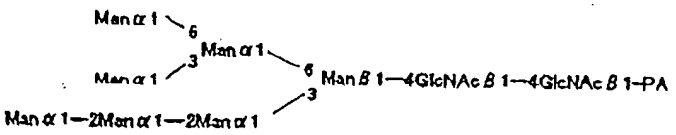
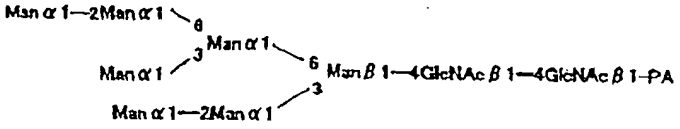
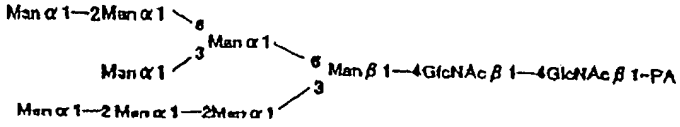
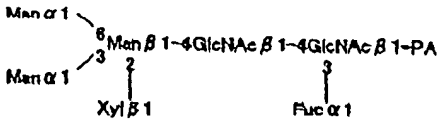
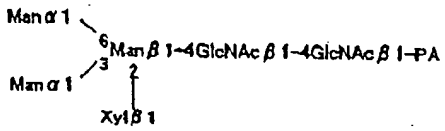
Peak8-4

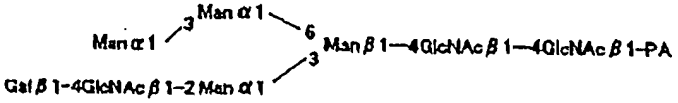
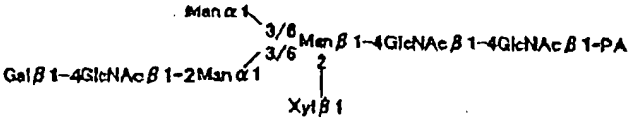
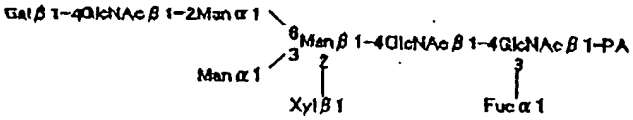
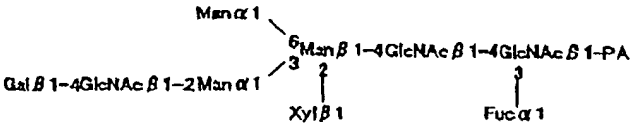
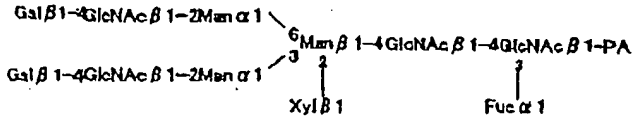
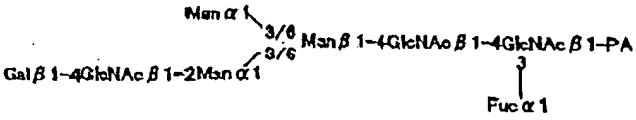
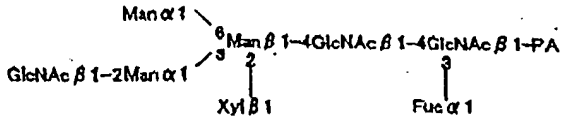
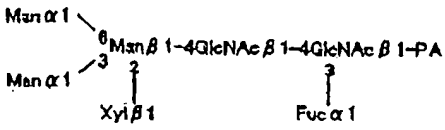


Retention time (min)

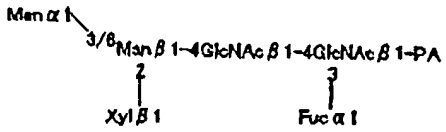
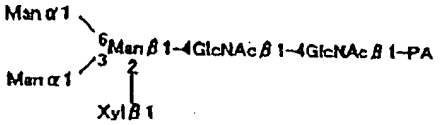
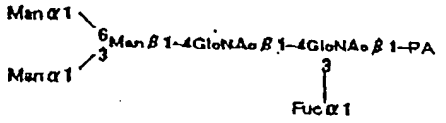
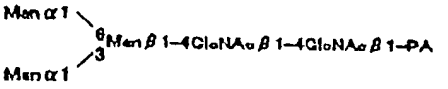
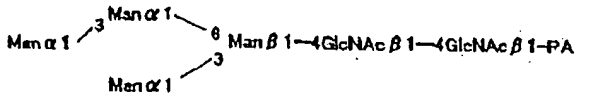
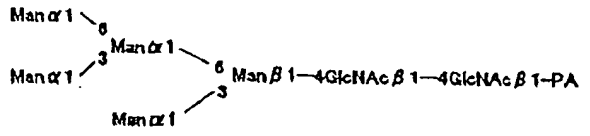
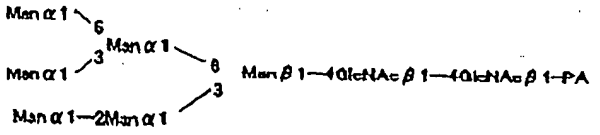
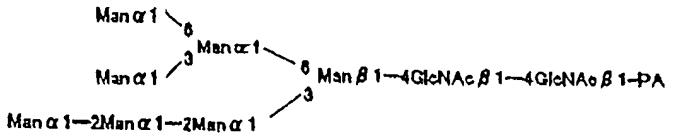
Abbreviation	Structure
GalGNM5 (1.46%)	
GalGNM3X (2.95%)	
GalGN(α1, 6)M3FX (1.29%)	
GalGN(α1, 3)M3FX (2.93%)	
GalGN2M3FX (2.72%)	
GNM3FX (3.63%)	
GNM3FX (6.70%)	
GN2M3FX (19.4%)	
GNM5 (4.44%)	

Glycan structures of hGT transgenic tobacco plants

Abbreviation	Structure
GN2M3FX (19.4%)	
M5A (5.90%)	
M6B (3.05%)	
M7B (3.34%)	
M7A (2.00%)	
M8B (3.47%)	
M3FX (34.6%)	
M3X (1.08%)	

Abbreviation	Structure
GalGNM4 (3.24%)	
GalGNM3X (3.79%)	
GalGN(α1, 6)M3FX (5.76%)	
GalGN(α1, 3)M3FX (13.2%)	
Gal2GN2M3FX (3.68%)	
GalGNM3F (3.31%)	
GNM3FX (5.91%)	
M3FX (19.5%)	

Glycan structures of hGT transgenic tomato plants

Abbreviation	Structure
M2FX (2.71%)	
M3X (4.87%)	
M3F (7.11%)	
M3 (1.76%)	
M4 (1.17%)	
M5A (0.391%)	
M6B (0.167%)	
M7B (23.5%)	

(continued)

List of results of sugar chain structural analysis of wild type tobacco (Nicotiana tabacum SR1)

RP-HPLC	SF-HPLC	Mass Spectrum (g/mol)	Calculated Mass (g/mol)	Deduced Sugar Chain Structure	Abbreviation	Proportion (%)
4	b	973.50	972.94	Man2FucGlcNAc2-PA	M2F	0.29
	e	1268.13	1267.19	Man3XylFucGlcNAc2-PA	M3FX	34.59
	g	1800.61	1799.64	Man8GlcNAc2-PA	M8A	4.04
5	a	943.33	942.91	Man1XylFucGlcNAc2-PA	MFX	0.65
	e	1135.98	1135.08	Man3FucGlcNAc2-PA	M3F	3.14
	k	1962.96	1961.78	Man9GlcNAc2-PA	M9A	2.52
6	a	1105.43	1105.05	Man2XylFucGlcNAc2-PA	M2FX	5.59
	d	1470.32	1470.38	GlcNAcMan3XylFucGlcNAc2-PA	GN1M3FX	34.13
	e	1637.71	1637.50	Man7GlcNAc2-PA	M7B	0.27
7	c	1176.68	1176.13	GlcNAcMan2FucGlcNAc2-PA	GN1M2F	0.44
	d	1192.84	1192.13	GlcNAcMan3GlcNAc2-PA	GN1M3	0.18
	e	1339.01	1338.27	GlcNAcMan3FucGlcNAc2-PA	GN1M3F	6.73
	f	1517.27	1516.41	GlcNAcMan5GlcNAc2-PA	GN1M5A	0.14
8	a	989.24	988.94	Man3GlcNAc2-PA	M3	0.96
	b	1121.48	1121.05	Man3XylGlcNAc2-PA	M3X	0.55
	c	1151.76 1308.96	1151.08 1308.24	Man4GlcNAc2-PA GlcNAcMan2XylFucGlcNAc2-PA	M4B GN1M2FX	0.46 2.04
	d	1325.07	1324.24	GlcNAcMan3XylGlcNAc2-PA	GN1M3X	0.44
	e	1314.06	1313.22	Man5GlcNAc2-PA	M5A	1.87
9	e	1396.46	1395.32	GlcNAc2Man3GlcNAc2-PA	GN2M3	0.14
	f	1528.81	1527.43	GlcNAc2Man3XylGlcNAc2-PA	GN2M3X	0.31
10	b	1325.05	1324.24	GlcNAcMan3XylGlcNAc2-PA	GN^{1}M3X	0.52